1.1 Biofilm: Definition and basic concepts

In fact, there has been an explosion of studies examining microbial biofilms in the last 20 years, which have been accompanied by the development and improvement of the techniques that revolutionized our understanding of biofilms; but despite the several advantages brought about by the new techniques, a very simple question still remains: what is a biofilm?

The term biofilm is self-explanatory, but even for biofilm researchers, its definition remains controversial for many reasons. For example, film semantically implies a continuous and relatively thin layer, but many biological structures regarded as biofilms are neither continuous nor thin (Lewandowski and Beyenal 2007). Moreover, research on biofilms has developed into interdisciplinary work, and scientists involved are from different research fields that leads to individual judgements regarding the professional area. Consequently, it can be said that there are almost as many definitions as there are scientists working in the biofilm field. Facing the problematic definition, Wimpenny (2000) listed the types and descriptions of microbial systems that are related to biofilms but, as a result of these diverse definitions, still left divergences in the application of the terminology.

Despite the difficulties in defining biofilm, and the diversity of pathways utilized to make a biofilm documented, the past decades have revealed common phenotypes conserved among biofilms. Thus, observing similarities among very different biofilms will likely teach us much. In this chapter, it used a simple and widely accepted definition which says that biofilms are microbial communities formed by microorganisms attached to a surface and enclosed in a matrix of extracellular polymeric substances (EPS) (Donlan and Costerton 2002; Stoodley et al. 2002; Harrison et al. 2005; Huq et al. 2008). As microbial communities, biofilms are assemblages of diverse species occupying the same, functional discrete environment and have a complex level of organization with a distinctive structure, own activities and laws, which depend on the relationships between their constituents (Wimpenny 2000).

In general, for the development of a biofilm, the cell leaves its planktonic condition and attach to a surface and/or other cells within an exopolymeric matrix. In a biofilm, the structures of individual cells are not significantly altered, but the individuals become organized into a complex structure and display novel characteristics and phenotypes (Harding et al. 2009). The physical proximity of other cells promotes synergistic interactions and aid to microbial cells in numerous aspects of their life cycles. These benefits may include increased tolerance to chemical, biological and physical stresses; efficient capture of nutrients; enhanced cell to cell communication; and colonization of host tissues (Lewis 2001; Mahmoud and O'Toole 2001). A typical bacterial biofilm development model can be described in five main stages: (1) adsorption, association or
initial attachment of a single cell to a surface, (2) adhesion, (3) microcolony formation, (4) maturation and (5) dispersal.

The third stage of biofilm development in which cells form microcolonies is characterized by the production of EPS. EPS can represent 50–90% of the total organic matter of biofilms and are responsible for binding cells and other particulate materials together (cohesion) and to the surface (adhesion), that is, providing the structural support for the biofilm maturation (Allison 2003). Polysaccharides are characteristic components of EPS, but its chemistry is complex and in general also comprises proteins, nucleic acids, lipids, phospholipids and humic substances. Although polysaccharides have been well studied, the literature suggests a large variety, but uncharacterized, of components produced by different species under different growth conditions (Sutherland 2001). Beyond mechanical stability, EPS protect biofilm against adverse conditions and biocides and also permit the development of microconsortia, concentration gradients, retention of extracellular enzymes, convective mass transport through channels, easy horizontal gene transfer, a matrix for exchange of signalling molecules and light transmission into the deeper layers of the biofilm structure (Flemming 2002). Biofilm formation at the interface between a solid substratum and a liquid is a common phenomenon in natural, medical and industrial environments. In water distribution systems, it is estimated that 95% of microbial biomass is in biofilms (Momba et al. 2000); thus, biofilms are considered a main reservoir of pathogens and a great threat to safe drinking water.

Despite difficulties defining the limits of the group, mycologists have defined fungi as ‘eukaryotic, spore-producing, achlorophyllous organisms with absorptive nutrition that generally reproduce both sexually and asexually and whose usually filamentous branched somatic structures, known as hyphae, typically are surrounded by cell walls’ (Alexopoulos et al. 1996). Based on their lifestyle, fungi are characterized by heterotrophic nutrition and cosmopolitan distribution (Kendrick 1992). As a matter of didactic and a practical approach to classification, fungi have been divided into groups based on their morphology, that is, filamentous fungi (or moulds), yeasts and mushrooms.

As a diverse and dynamic group, fungi are involved in many activities that affect human both in a good and bad way. In general, the single most important role that fungi play is not specifically identified yet, but fungi are the most important agent of decay on Earth (Alexopoulos et al. 1996) and play a predominant role in recycling organic matter in the environment. Fungi are often observed on decaying foodstuff on which some fungi produce toxins (mycotoxins); many of them are plant and human pathogens. In addition, fungi are used to produce commercial products such as antibiotics (e.g. penicillin), organic acids (e.g. citric acid), industrial alcohol (e.g. biofuel) and enzymes (e.g. amylases). Moreover, fungi are also used in food industry for the production of a diverse range of important foodstuffs such as bread, beer, cheese, meats and soy sauce (Paterson and Lima 2005).

1.2 Fungi and fungal biofilms

1.2.1 Fungi

Fungi are a ubiquitous and diverse group of organisms belonging to the kingdom Fungi which was first considered as the fifth kingdom by Whittaker (1959). According to the most recent classification, this kingdom comprises 1 subkingdom, 7 phyla, 10 subphyla, 35 classes, 12 subclasses and 129 orders (Hibbett et al. 2007). It has been estimated that 1.5 million species exist worldwide and about only 120,000 species have been described to date (Kirk et al. 2001).

1.2.2 Filamentous fungal biofilms

Bacterial and yeast biofilms have been greatly studied in the last 20 years (Chandra et al. 2001; Kumamoto and Vinces 2005; De Beer and Stoodley 2006; Walker and Marsh 2007; Shi and Zhu 2009). Consequently, there are well-defined models, criteria and phenotypes for characterizing bacterial and yeast biofilms. On the other hand, a lack of information about filamentous fungal (ff) biofilms still remains, though filamentous fungi are extremely adapted to grow on surfaces (Jones 1994).

Indeed, the term ‘biofilm’ is rarely applied to filamentous fungi, but there have been several descriptions indicating that filamentous fungi grow as biofilms in different medical, environmental and industrial settings (Anaissie et al. 2003; Gutierrez-Correa and...
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Villena 2003; Mowat et al. 2007; Mowat et al. 2008). Harding et al. (2009) proposed criteria for biofilm formation by filamentous fungi which are grouped in (i) structural features such as complex aggregated growth, surface-associated growth of cells and secreted extracellular polymeric matrix and (ii) altered gene expression resulting in phenotypic changes that include enhanced tolerance to antimicrobial compounds or biocide changes in enzyme or metabolite production and/or secretion physiological changes. The reports previously mentioned demonstrate that the structural and phenotypic criteria can be fulfilled by some filamentous fungi.

Based on already published descriptions for filamentous fungi and drawing from bacterial and yeast models, Harding et al. (2009) proposed a preliminary model for ff biofilm formation (Figure 1.1) in which biofilm development follows six main steps including:

1. Propagule adsorption: deposition of spores or other propagules such as hyphal fragments or sporangia.
2. Active attachment to a surface: includes secretion of adhesive substances by germinating spores and active germlings.
3. Microcolony formation: production of a polymeric extracellular matrix that allows the growing colony to adhere tenaciously to the substrate.
4. Initial maturation: formation of compacted hyphal networks or mycelia and hypha–hypha adhesion and the formation of water channels via hydrophobic repulsion between hyphae or hyphal bundles.
5. Maturation: it is characterized by the formation of reproductive structures.
6. Dispersal or planktonic phase: involves spore dispersal or release or the dispersal of biofilm fragments.

Figure 1.1 ff biofilm formation model: (i) adsorption, (ii) active attachment, (iii) microcolony I (germling and/or monolayer), (iv) microcolony II (mycelial development, hyphal layering, hyphal bundling), (v) development of the mature biofilm and (vi) dispersal or planktonic phase. Reproduced with permission from Elsevier, Harding et al. (2009). © Elsevier

An investigation in Aspergillus niger biofilms and pellets showed that these types of growth have two main structural differences: only biofilms exhibited surface heterogeneity and interstitial voids with well-defined channels. Other differences include the growth direction, that is, biofilm growth was mainly vertical, and a specific biomass distribution as well (Villena et al. 2010). This same work reported that structural differences were associated with a differential physiological behaviour regarding enzymatic production.

Compact hyphal balls of Aspergillus fumigatus were characterized as biofilms once they presented production of an extracellular polymeric matrix, differential gene expression and differential sensitivity to antifungal drugs (Beauvais et al. 2007; Mowat et al. 2008). Increased resistance against biocides is one characteristic often described for biofilms, and several studies have been carried out to evaluate the in vitro susceptibility of pathogenic fungal biofilms (Jabra-Rizk et al. 2004; Bonaventura et al. 2006; Seidler et al. 2008). Still about A. fumigatus, extracellular DNA was recently identified as an important component of its biofilm. The extracellular DNA, either originated in fungal autolysis or when externally supplied, presents a high affinity for nucleic acids to the cell wall of A. fumigatus (Krappmann and Ramage 2013; Rajendran et al. 2013; Shopova et al. 2013). These studies showed that extracellular DNA promotes adhesion of the conidia in the initial phase of biofilm formation, triggers polysaccharide formation and becomes incorporated in the biofilm, thereby shaping its overall structure.

In environmental studies, ff biofilm descriptions have been reported as well. For example, in historical monuments, ff biofilms were described forming
complex consortia with cyanobacteria and algae resulting in biowereathering of the substrata and thus causing biodeterioration (Grbić et al. 2010). An investigation in microbial communities on the surfaces and within the painting layers of mural paintings of a church showed that the main biofilm formers were microscopic fungi belonging to the genera Acremonium, Aspergillus, Cladosporium and Fusarium (Gorbushina et al. 2004). Müller et al. (2001) also described microbial colonization of the surface of historic glass panels ageing from 30 to 600 years and found a heterogeneous colonization with filamentous fungi as the dominant group. Phylogenetic analysis revealed that in acid mine drainage biofilms, the majority of the sequences belonged to fungi (Baker et al. 2009). All these reports have in common natural ff biofilm growing in oligotrophic environments, showing high tolerance against adverse factors (e.g. temperature and dryness) and intimate interaction with other microorganisms such as bacteria and algae.

Hydrophobicity is related to many factors of fungal life and is crucial for fungal survival and adaptation; ff are known to produce hydrophobins which are small proteins localized on the outer surface of their cell walls. These proteins form an amphipathic membrane whose hydrophobic side is exposed to the exterior, while the hydrophilic surface is bound to the cell wall polysaccharides and confer water-repellent properties (Whiteford and Spanu 2002). Hydrophobic interactions are of major importance in the firm adhesion of diverse microorganisms to water–solid interfaces (Donlan and Costerton 2002). Although the hydrophobic effect has been considered to be non-specific, it is known that fungal–bacterial biofilms can be mediated by hydrophobic and electrostatic interactions wherein the fungal cell acts as a surface for bacteria to be attached on (Morales and Hogan 2010).

Siqueira and Lima (2012) reported that within Penicillium expansum and Penicillium brevicompactum biofilms, the hyphae projected out of the denser hyphae layer, and exposed to the outer inner of the biofilm, were those that presented higher hydrophobicity. This observation is in line with the model for the formation of fungal aerial structures, which postulate that hyphae are cover by a hydrophobin film with its hydrophobic side exposed to the air (Wösten et al. 1994), but with a particular feature: the hydrophobic hyphae are still in contact with the liquid medium. The most hydrophobic hyphae seem to be projected out of the biofilm core to create a differentiated mycelial zone, which can be associated with further interactions in aquatic environments. It is also important to know that environmental conditions such as temperature, nutrient source and humidity can affect hydrophobicity (Smits et al. 2003).

The importance of fungal biofilm phenotype concept in medical and industrial mycological research was recently reported by Ramage et al. (2011). These authors described schematically Aspergillus biofilm development (Figure 1.2) and discussed morphological, physiological and molecular features related to both fungal virulence and enzymatic production. Nonetheless, the improvement and standardization of suitable methods for laboratorial studies of filamentous fungi biofilms are few.

Intending to investigate the capability of biofilm formation and characterize morphologically and physiologically, Siqueira et al. (2013) studied the biofilm of Aspergillus sp. (section Nigri), Aspergillus sp. (section Flavi), Alternaria sp., Botrytis sp., Cladosporium sp. and Penicillium sp., isolated from biofilms in a water system. Each fungus presented a different pattern of biofilm development, spore adhesion, monolayer and EPS production in all fungal species. Moreover, characteristics of spores and culture conditions play an important role in filamentous fungal biofilm kinetics and must be taken into consideration for further studies in this area.

Although filamentous fungi have been commonly recovered from drinking water and are often listed as integrant of microbial water biofilms (Kerr et al. 2003), ff biofilms in drinking water system have been disregarded, and the focus of most research has been put on bacterial biofilms, especially on those linked with water-related illness (Huq et al. 2008). Drinking water systems are undoubtedly complex environments wherein bacteria, fungi, protozoa, viruses and algae cohabit and interact. Each microorganism plays its own roles and should not be underestimated, either as a potential threat to human health or as functional part of this unique ecological niche.

The study of biofilms in drinking water systems is prone to errors since the main drawbacks in this area are related to the variation in scientific methodology (Berry et al. 2006; Hageskal et al. 2009). Others features
such as representativeness of samples, heterogeneity of environment and sort of techniques applied must be taken into consideration (Figure 1.3). For example, different sources of water and variable time of exposure are commonly found in studies of ff biofilms in water systems, and comparisons between results become difficult. Additionally, in water networks, the collection of pipes is not easy since their removal would be necessary; consequently, in situ approaches are scarce. Pilot systems in laboratory are used instead.

Understandings of ff biofilm development, dynamics and interactions require further research for a better clarification about this naturally occurring growth form, thereby unraveling its impact and role in medical, industrial and environmental areas.

1.2.3 Methods to study fungal biofilms

Research on biofilms is an interdisciplinary work in which researchers from different areas are involved. The field of interest will determine the specific approaches to be applied and may include microscopical, microbiological, molecular biological, (bio)chemical and/or physical methods. The following scheme (Figure 1.4) summarizes research fields and techniques applied in biofilm studies.
Figure 1.4  Research fields and analytical techniques applied in biofilm research. AAS, atomic absorption spectrometry; AFM, atomic force microscopy; ATR, attenuated total reflectance; CE, capillary electrophoresis; CLSM, confocal laser scanning microscopy; DGGE, denaturing gradient gel electrophoresis; FFF, field-flow fractionation; FISH, fluorescent in situ hybridization; GC, gas electrophoresis; GE, gel electrophoresis; GFP, green fluorescent protein; IR, infrared; LC, liquid chromatography; NMR, nuclear magnetic resonance; PCR, polymerase reaction; PFGE, pulsed-field gel electrophoresis; SEC, size exclusion chromatography; SEM, scanning electron microscopy; ST XM, scanning transmission X-ray microscopy. Reproduced with permission from John Wiley & Sons, Ltd., Denkhaus et al. (2007). © Springer

References


